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ENDOCRINE RESPONSES TO EXOGENOUS BOMBESIN AND GASTRIN RELEASING PEPTIDE IN CONSCIOUS CALVES

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SUMMARY

1. The effects of I.V. infusions of synthetic amphibian bombesin and porcine gastrin releasing peptide (GRP), at a dose of 5 pmol/kg.min for 30 min, have been investigated in conscious calves 3–6 weeks after birth.

2. The protocols produced a closely similar rise in the bombesin-like immunoreactivity of the arterial plasma of 208 ± 14 pmol/l (bombesin) and 210 ± 32 pmol/l (GRP) which fell exponentially with a half-life of about 3 min when the infusions were terminated.

3. Neither peptide produced a discernible change in mean heart rate or aortic blood pressure, or in the mean arterial plasma concentrations of enteroglucagon, gastric inhibitory peptide (GIP), gastrin or cholecystokinin (CCK).

4. GRP, but not bombesin, produced a small but significant rise in the mean plasma somatostatin concentration.

5. Both peptides produced a significant rise in mean plasma pancreatic glucagon and pancreatic polypeptide concentration and proved to be exceptionally potent insulinotropic agents. These responses were associated with a rise in plasma glucose concentration which could not be attributed to a direct action of GRP on the liver.

6. The distribution of bombesin-like immunoreactivity in the gastrointestinal tract was consistent with the findings of other workers who have concluded that it is restricted to nerve terminals. However, our other findings show that GRP is capable of acting as a true hormone.

INTRODUCTION

Stimulation of the peripheral ends of the splanchnic nerves causes an abrupt and substantial rise in bombesin-like immunoreactivity in the arterial plasma and intestinal lymph of adrenalectomized 3–5 week old calves (Bloom & Edwards, 1982). We now report the results of a study which was designed to elucidate the physiological consequences of this response. These were investigated by assessing the effects of I.V. infusions of bombesin and of a synthetic gastrin releasing peptide (GRP), which shares the same minimal active sequence of amino acids with bombesin in conscious calves. Infusions of these peptides at a dose of 5 pmol/kg.min for 30 min resulted in a closely similar rise in plasma bombesin-like immunoreactivity to that previously

observed during splanchnic nerve stimulation at 40 Hz for 1 s at 10 s intervals for 10 min (Bloom & Edwards, 1982). Both peptides proved to be exceptionally potent insulinotropic agents when infused at this dose and also caused a significant rise in the concentration of certain other peptides in the circulating plasma.

Some of these results have been published previously in a preliminary form (Bloom, Edwards & Ghatei, 1983).

METHODS

Animals

Pedigree Jersey calves were obtained from local farms shortly after birth and used at ages ranging from 24 to 44 days (29–39 kg body weight). The animals were kept in individual pens and maintained on a diet of either cow's milk or artificial milk (Easy-mix Volac; Volac Ltd.) at a rate of 2–4 l/day. Food was invariably withheld for at least 6 h prior to surgery and 16 h before each experiment.

Experimental procedures

Preparatory surgery involved the insertion of a narrow-bore Polythene catheter via the saphenous artery so that the tip lay in the abdominal aorta. This procedure was carried out under general anaesthesia, induced with chloroform (Chloroform SLR; Fisons) and maintained with halothane (May & Baker; *circa* 2% in oxygen). The arterial catheter was used subsequently to monitor aortic blood pressure and heart rate and for collection of arterial blood samples.

Experiments were carried out at least 24 h after surgery and were invariably started at about 10.00 a.m. in order to minimize any possible diurnal variations. Just before each experiment a Braunula cannula was inserted into one or other jugular vein to provide a conduit for I.V. infusions. On each occasion pure synthetic amphibian bombesin (Bachem, Torrance, CA, U.S.A.) or pure synthetic porcine gastrin releasing peptide (GRP, kind gift of Professor N. Yanaihara, Shizuoka Japan) was dissolved in an appropriate volume of sterile physiological saline for I.V. infusion at a dose of 5 pmol/kg . min for 30 min and a volume of 1 ml/min.

Several calves, in which both splanchnic nerves had been cut under general anaesthesia 2–3 weeks previously, were used to investigate the hepatic responses to intramesentric infusions of GRP. Each of these animals was anaesthetized by means of an I.V. infusion of α-chloralose (70–80 mg/kg). Both adrenal veins were then ligated and the pancreas was removed. Catheters were inserted into the femoral arteries and veins and into a convenient hepatic vein. The latter manoeuvre necessitated thoracotomy followed by insertion of a hepatic cannula directly through the wall of the posterior vena cava, immediately above the diaphragm, so that the tip could be positioned several centimetres within one or other of the main hepatic venous channels. Thereafter respiration was maintained by intermittent positive pressure ventilation and aortic blood pressure, P_{O_2}, P_{CO_2} and blood pH were continually monitored.

In addition samples of liver, pancreas, kidney, lung and various regions of the gastrointestinal tract were excised from four control calves of the same age under barbiturate anaesthesia (Sagatal, May & Baker, 30 mg/kg I.V.) for subsequent extraction and measurements of bombesin-like immunoreactivity. These tissue samples were frozen immediately after collection by immersion in liquid nitrogen and sequestered at −40 °C. They were then weighed, diced and placed in 0·5 M-acetic acid at 100 °C. After 10 min the samples were cooled and stored at −20 °C until the supernatant was assayed.

Estimations

Samples of arterial blood were collected at intervals for peptide and glucose measurements. Aliquots destined for peptide assays were collected into heparinized tubes containing aprotinin (1000 K.I.U/ml blood) and centrifuged without delay at +4 °C; the plasma was subsequently stored at −20 °C. Glucose was measured by means of a Mark II Beckman Glucose Analyzer.

All peptide hormones were measured by radioimmunoassay (Bloom & Long, 1982). Pancreatic glucagon was measured using an antiserum relatively specific for pancreatic glucagon which was C-terminal reacting (Assan & Slusher, 1972) and gave zero values in human plasma after total pancreatectomy, even after arginine infusions (Barnes & Bloom, 1976), reacting less than 0·1% with pure porcine enteroglucagon (glicentin). Enteroglucagon was estimated by subtracting the

immunoreactivity attributable to pancreatic glucagon from the total obtained using a non-specific glucagon antibody. Pure glicentin was fully measured by this assay (Ghatei & Bloom, 1981). Insulin and pancreatic polypeptide (PP) were also measured by radioimmunoassays that we have employed routinely in the past (Albano, Ekins, Maritz & Turner, 1972; Adrian, Bloom, Bryant, Polak, Heitz & Barnes, 1976).

GRP or mammalian bombesin-like immunoreactivity was measured by an antiserum raised to synthetic lys^3-bombesin conjugated with glutaraldehyde to bovine serum albumin in rabbits. The radioactive assay label was prepared, using a tyr^5-bombesin C-terminal nonapeptide analogue, by chloramine-T oxidation and ^{125}I. This assay detects bombesin and pure porcine GRP with equal potency and is capable of distinguishing changes of 0·4 fmol/assay tube of tissue extract and 5 pmol/l of plasma bombesin-like immunoreactivity with 95% confidence (Ghatei, 1982).

Gastrin was estimated using an antibody raised to gastrin-17 in rabbits (Bryant & Adrian, 1982). The assay fully detected gastrin-34 but showed less than 1% cross-reactivity with cholecystokinin (i.e. CCK-8 and CCK-33). CCK was measured using an antiserum raised to CCK-octapeptide in rabbits which showed 100% cross-reaction with gastrin-17 and -34 and CCK-33 and -39. Total CCK- and gastrin-like immunoreactivity was measured and gastrin immunoreactivity (specifically detected as described above) subtracted from the sum of the two types. This assay system showed no rise in plasma gastrin after a meal in animals that had previously undergone gastric antrectomy, whereas the rise of CCK was unaltered. Chromatographic analysis by gel-permeation showed that CCK-like immunoreactivity eluted in two major peaks (Adrian & Bacarese-Hamilton, 1982), one coinciding with the position of CCK-8 (approximately 60% of the total) and the other co-eluting in the position of CCK-33 and -39 (approximately 40% of the total).

Gastric inhibitory peptide (GIP) was measured using an antiserum raised to the pure natural porcine peptide in rabbits. Natural GIP was iodinated with ^{125}I using lactoperoxidase. The assay also detected big GIP but did not show any cross-reaction with glucagon, secretin or vasoactive intestinal polypeptide (VIP). Differences of 3 pmol/l plasma could be detected between individual samples with 95% confidence (Sarson, Bryant & Bloom, 1980). Somatostatin was estimated using an antiserum raised in a rabbit to ovine somatostatin-14 The tyr-11 analogue was iodinated with ^{125}I using lactoperoxidase. The assay detected differences in plasma somatostatin between individual samples of 4 pmol/l with 94% confidence. It fully cross-reacted with somatostatin-28 but reacted poorly or not at all with fragments or shortened analogues (O'Shaugnessy, 1982).

Statistical analyses were made according to the methods of Snedecor & Cochran (1967).

RESULTS

Continuous intravenous infusions of either pure synthetic amphibian bombesin (bombesin) or of pure synthetic porcine GRP at a dose of 5 pmol/kg . min for 30 min in conscious 3–6 week old calves increased the mean bombesin-like immunoreactivity of the arterial plasma by closely similar amounts (208 ± 14 and 210 ± 32 pmol/l respectively; Fig. 1*A*). The duration of the infusion sufficed to produce a plateau in mean bombesin-like immunoreactivity in arterial plasma and it was found to disappear exponentially in each group, with a half-life of about 3 min, during the 10 min period after the infusions were terminated (Fig. 1*B*). Both heart rate and aortic blood pressure were monitored continuously during each experiment and no change in either parameter was detected in response to either peptide (Fig. 1*A*).

Both peptides produced an abrupt rise in mean arterial plasma pancreatic glucagon concentration, which was associated with a small rise in mean arterial plasma glucose concentration in both groups (Fig. 2). However, there was a much more substantial rise in the mean plasma insulin concentration, which had increased by 570 ± 120 pmol/l (bombesin) and 556 ± 110 pmol/l (GRP) at 10 min. Both responses were transient and the mean plasma insulin concentration of both groups fell thereafter during the remainder of the infusion. This fall was less pronounced in the

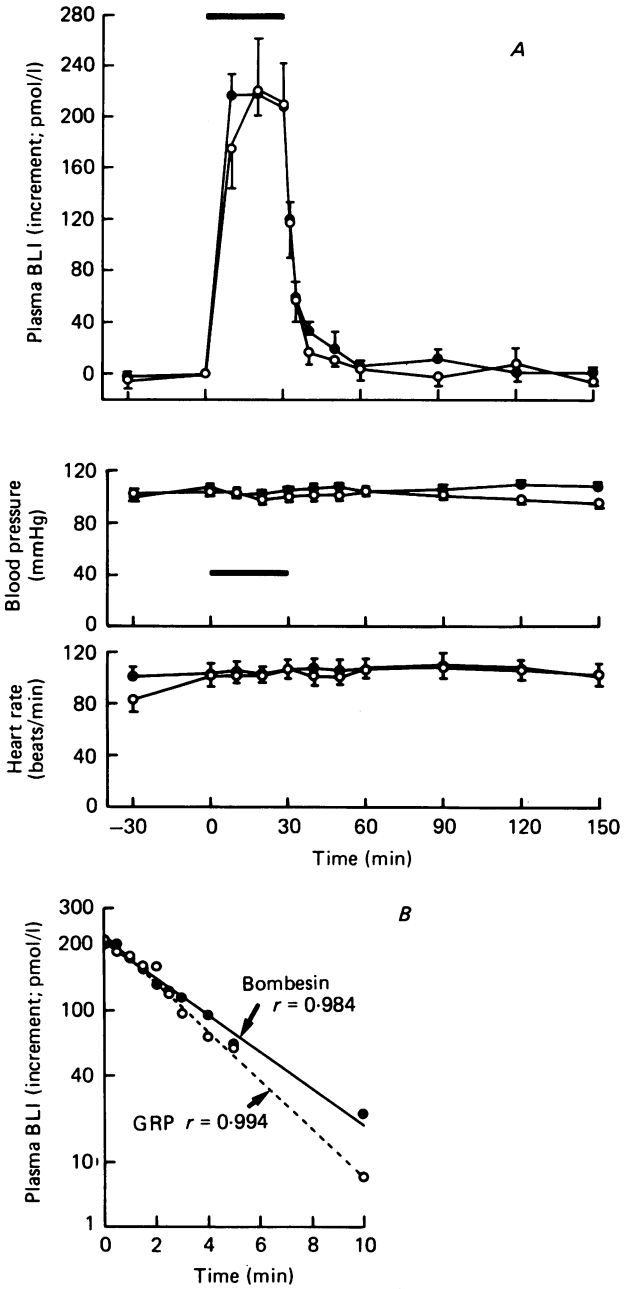

Fig. 1. *A*, changes in mean bombesin-like immunoreactivity (BLI) in arterial plasma, aortic blood pressure and heart rate in response to I.V. infusions of either amphibian bombesin (●, $n = 8$) or mammalian GRP (○, $n = 8$) in conscious 3–6 week old calves at a dose of 5 pmol/kg . min for 30 min. Horizontal bar: duration of infusion. Vertical bars: S.E. of each mean value. Absolute mean BLI values at time = 0 : 40 ± 8 pmol/l (bombesin infusions), and 52 ± 16 pmol/l (GRP infusions). *B*, the changes in mean arterial plasma BLI in the two groups during the 10 min immediately after infusions were discontinued, using an expanded semilog scale. Bombesin infusions: $t_{\frac{1}{2}} = 3{\cdot}7$ min; GRP infusions: $t_{\frac{1}{2}} = 2{\cdot}6$ min.

group infused with GRP than in that given bombesin and the mean incremental value in the former group at 30 min (230 ± 73 pmol/l) was significantly higher than the corresponding value in the latter (108 ± 37 pmol/l; $P < 0.02$). In both groups the mean plasma insulin concentration had subsided to within the normal range 20 min after the infusion had been discontinued.

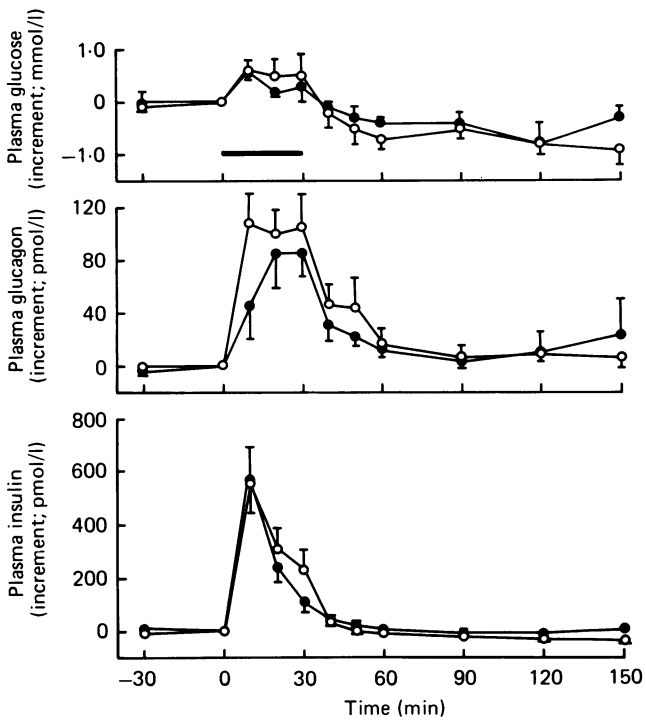

Fig. 2. Comparison of the changes in mean arterial plasma glucose, pancreatic glucagon and insulin concentration in conscious 3–6 week old calves in response to I.V. infusions of either amphibian bombesin (●, $n = 8$) or mammalian GRP (○, $n = 8$) at a dose of 5 pmol/kg for 30 min. Horizontal bar: duration of infusion. Vertical bars: S.E. of each mean value. Absolute values at time = 0 before bombesin infusions: glucose 4·1 ± 3 mmol/l, glucagon 23 ± 5 pmol/l, insulin 27 ± 5 pmol/l. Before GRP infusions: glucose 4·7 ± 0·1 mmol/l, glucagon 18 ± 5 pmol/l, insulin 45 ± 10 pmol/l.

Neither peptide produced any significant change in the mean concentration of GIP or enteroglucagon in the arterial plasma (Fig. 3). In contrast, both elicited a steady and closely similar rise in mean arterial plasma PP concentration, which had increased to peak incremental values of 57 ± 38 pmol/l (bombesin) and 53 ± 10 pmol/l (GRP) at 30 min and subsided steadily thereafter (Fig. 3). In spite of the biological potency of these peptides, as evidenced both by their intense insulinotropic effects and the release of PP they produced, there was no significant change in either mean plasma gastrin or CCK concentration in either group (Fig. 4).

Measurements of the changes in mean plasma somatostatin-like immunoreactivity during these experiments revealed a significant difference between the two peptides. Thus, bombesin failed to produce any detectable change at this dose whereas GRP

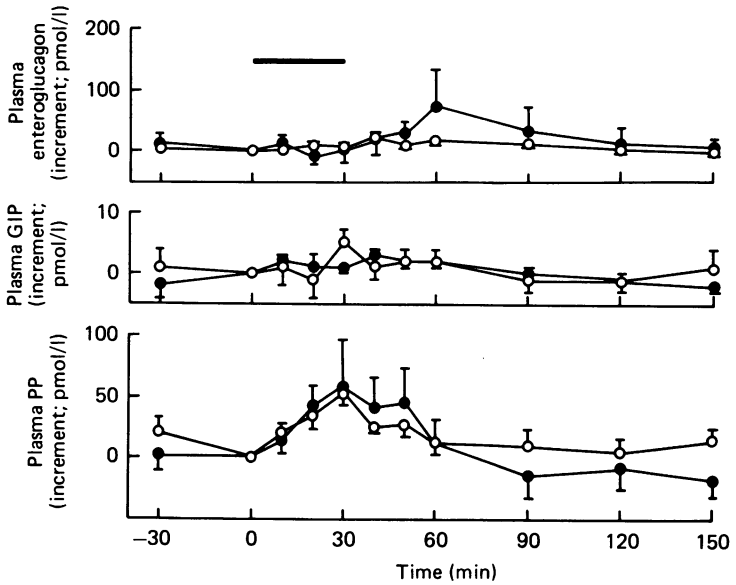

Fig. 3. Comparison of the changes in mean arterial plasma enteroglucagon GIP and PP concentration in conscious 3–6 week old calves in response to I.V. infusions of either amphibian bombesin (●, $n = 8$) or mammalian GRP (○, $n = 8$) at a dose of 5 pmol/kg.min for 30 min. Horizontal bar: duration of infusion. Vertical bars: S.E. of each mean value. Absolute values at time = 0 before bombesin infusions: enteroglucagon 89±29 pmol/l, GIP 19±1 pmol/l, PP 127±15 pmol/l. Before GRP infusions: enteroglucagon 34±8 pmol/l, GIP 15±3 pmol/l, PP 85±10 pmol/l.

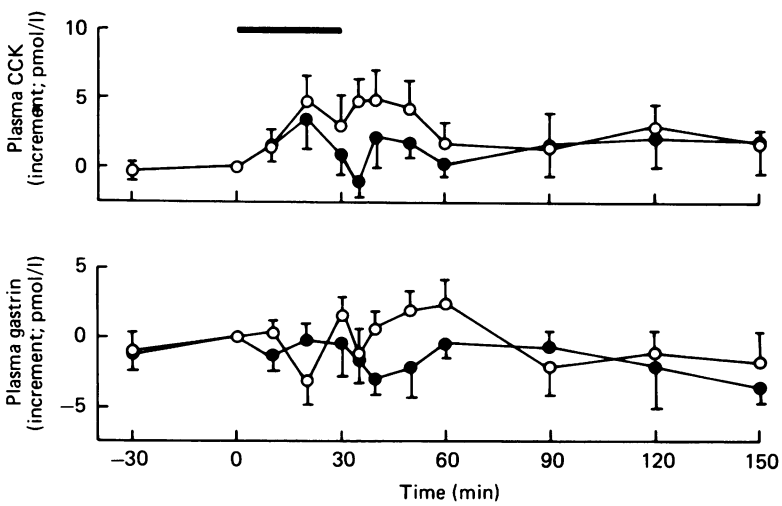

Fig. 4. Comparison of the changes in mean arterial plasma CCK-like and gastrin-like immunoreactivity in conscious 3–6 week old calves in response to I.V. infusions of either amphibian bombesin (●, $n = 8$) or mammalian GRP (○, $n = 8$) at a dose of 5 pmol/kg.min for 30 min. Horizontal bar: duration of infusion. Vertical bars: S.E. of each mean value. Absolute values at time = 0 before bombesin infusions: CCK 2·0±0·4 pmol/l, gastrin 9·8±1·5 pmol/l. Before GRP infusions: CCK 2·5±0·5 pmol/l, gastrin 11·3±6·1 pmol/l.

elicited a steady rise in mean plasma concentration of somatostatin-like immunoreactivity throughout the infusion (Fig. 5), and the absolute value at 30 min (77 ± 6 pmol/l) was significantly higher than the initial value (40 ± 13 pmol/l; $P < 0.05$).

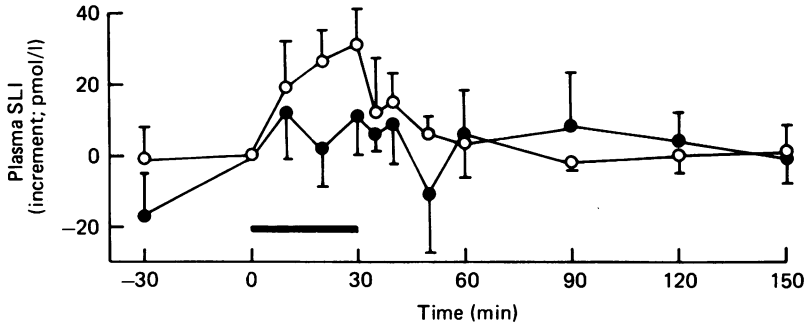

Fig. 5. Comparison of the changes in mean arterial plasma somatostatin-like immunoreactivity (SLI) in conscious 3–6 week old calves in response to I.V. infusions of either amphibian bombesin (●, $n = 8$) or mammalian GRP (○, $n = 8$) at a dose of 5 pmol/kg.min for 30 min. Horizontal bar: duration of stimulus. Vertical bars S.E. of each mean value. Absolute value at time = 0 before bombesin infusions: 56 ± 14 pmol/l. Before GRP infusions: 40 ± 13 pmol/l.

TABLE 1. Mean bombesin-like immunoreactivity in extracts of the smooth muscle and mucosa of different regions of the gastrointestinal tract in four 3–6 week old calves

	Bombesin-like immunoreactivity (pmol/g)		
Tissue	Muscle	Mucosa	
Duodenum	27·5 ± 4·4	8·7 ± 2·5	$P < 0.01$
Jejunum	34·0 ± 5·2	4·4 ± 2·6	$P < 0.01$
Ileum	31·3 ± 3·3	4·0 ± 0·4	$P < 0.01$
Colon	21·5 ± 0·4	4·2 ± 1·3	$P < 0.05$
Caecum	7·5 ± 1·9	2·6 ± 0·5	$P < 0.05$
Rectum	6·1 ± 1·7	3·8 ± 1·0	$P < 0.30$

P values relate to the differences between the mean values in the smooth muscle and the mucosa in each case.

Bombesin-like immunoreactivity in tissue extracts

Bombesin-like immunoreactivity was found to be present in greatest amounts throughout the gastrointestinal tract between the duodenum and the colon and in much greater quantities in the smooth muscle than in the mucosa in the duodenum, jejunum, ileum and colon (Table 1). There was also significantly more bombesin-like immunoreactivity in the smooth muscle of the caecum than in the mucosa of that viscus although the total amounts were much lower in both cases, as they were in the rectum. The differences between the values recorded in the colon and small intestine are so great as to suggest that the mucosal content might be attributable to contamination as the tissues were separated by crudely scraping the mucosa off

the muscle layers as expeditiously as possible. With the exception of the liver, in which mean bombesin-like immunoreactivity was found to be $7{\cdot}1 \pm 1{\cdot}5$ pmol/g, and the antrum of the abomasum (in which it was *circa* 7 pmol/g), much lower amounts were encountered in all other tissues that were assayed. These included the rumen and reticulum, the bladder, pancreas, kidney and lung. All these values refer to activity expressed as pmol/g wet weight of tissue.

Hepatic responses to GRP

The observation that the liver contained measurably higher bombesin-like immunoreactivity than other tissues prompted us to investigate the possibility that the

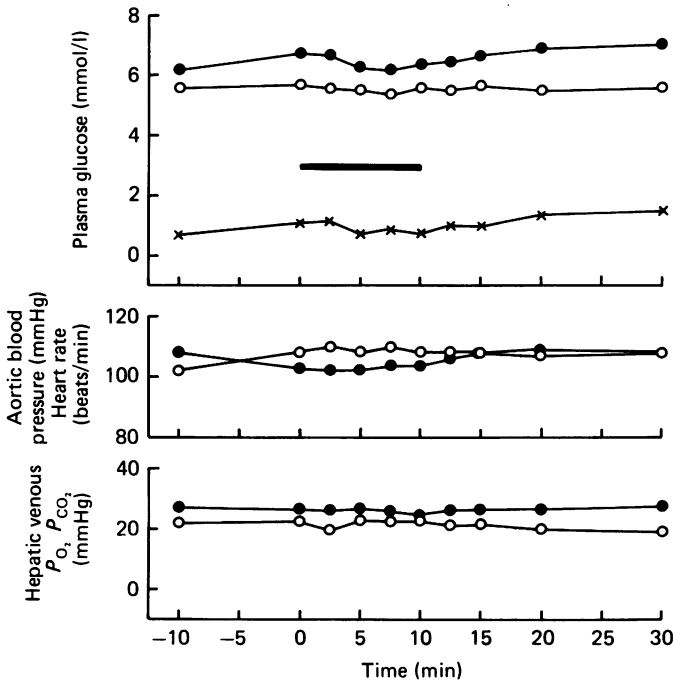

Fig. 6. Effects of an intraportal infusion of GRP (5 pmol/kg . min) for 10 min in a 6 week old calf under chloralose anaesthesia. Prior to the infusion both splanchnic nerves had been cut and both adrenal veins ligated. The pancreas had been extirpated and atropine administered at a dose of 0·2 mg/kg. Upper panel: changes in glucose concentration in hepatic venous effluent plasma (●), arterial plasma (○) and the hepatic arterio−venous difference (×). Centre panel: changes in aortic blood pressure (●) and heart rate (○). Lower panel: changes in hepatic venous P_{CO_2} (●) and P_{O_2} (○). Horizontal bar: duration of infusion.

hyperglycaemic response to exogenous GRP might be consequential upon a direct action of the peptide on the liver. This possibility was investigated in several calves of the same age under chloralose anaesthesia. In order to eliminate any indirect effects which might stimulate hepatic glycogenolysis the experiments were carried out in calves in which both splanchnic nerves had been cut and both adrenal veins ligated. In addition, the animals were pancreatectomized and given atropine (0·2 mg/kg)

10 min before GRP was infused. The experimental protocol involved the simultaneous collection of samples of arterial and hepatic venous effluent blood at intervals before, during and after a 10 min infusion of GRP via a mesenteric tributary of the portal vein at a dose of 5 pmol/kg . min. There were no significant changes in the concentration of glucose in either the arterial or hepatic venous plasma in any of these experiments and consequently no change in the hepatic arterio – venous difference in plasma glucose concentration. The aortic blood pressure was not apparently affected by GRP, and the P_{O_2}, and P_{CO_2} and the pH of both the arterial and hepatic venous blood were also unaffected by infusions of the peptide, indicating that it caused no detectable change in either hepatic blood flow or metabolism. The results of a typical experiment in this series are illustrated in Fig. 6. Precisely similar negative results were obtained in two experiments in which a higher dose of GRP (25 pmol/kg . min) was employed under identical conditions.

DISCUSSION

The rise in the mean bombesin-like immunoreactivity (BLI), which occurred in the circulating blood of these normal conscious calves, is probably within the physiological range because it reproduced that which occurs in response to splanchnic nerve stimulation in conscious adrenalectomized calves of the same age at an average frequency of 4 Hz (Bloom & Edwards, 1982). Furthermore, there was no detectable change in heart rate or blood pressure during these experiments, such as have been reported in other species by workers who have employed higher doses under less physiological conditions (Erspamer, Improta, Melchiori & Sopranzi, 1974; Melchiori, 1978), and the half-life of the peptide in the plasma was closely similar to that which has previously been reported in man (Ghatei, Jung, Stevenson, Hillyard, Adrian, Lee, Christofides, Sarson, Mashiter, Macintyre & Bloom, 1982).

The fact that no physiological function has yet been definitely ascribed to PP detracts from the interest that might otherwise be generated by the finding that it is released in response to physiological levels of BLI in the circulation. However, the observation that PP is released in response to both bombesin and GRP confirms the findings of several groups in other species (Lezoche, Vagni, Carlei, D'Alessandro, Mariani, Terenzi, Ricciuti & Basso, 1979; Taylor, Walsh, Carter, Wood & Grossman, 1979; MacDonald, Ghatei, Bloom, Track, Radziuk, Dupré & Mutt, 1981; Lezoche, Basso & Speranza, 1981; Ghatei *et al.* 1982). It may also account for the fact that a significant rise in mean plasma PP concentration occurs in response to splanchnic nerve stimulation at the same average frequency (4 Hz) in the presence of both α- and β-adrenoceptor blocking agents, because there is also a significant rise in mean plasma bombesin-like immunoreactivity under these conditions (S. R. Bloom, A. V. Edwards & M. A. Ghatei, unpublished observations).

It has been reported previously that infusions of bombesin in conscious dogs, at about the same dose as that employed in the present study, causes a rise in somatostatin-like immunoreactivity in the plasma (Schusdziarra, Rouiller, Harris, Pfeiffer & Unger, 1980). We failed to confirm that finding in the present study but the same dose of GRP did cause a significant rise in mean plasma somatostatin-like

immunoreactivity. This was the only instance in which the two peptides produced clearly different responses and it is interesting that the mammalian form proved effective and the amphibian form ineffective. A further difference between the present findings and those of others who have investigated the biological activities of bombesin (Bertaccini, Erspamer, Melchiorri & Sopranzi, 1974; Fender, Curtis, Rayford & Thompson, 1976; Konturek, Krol & Tasler, 1976; Basso, Lezoche & Speranza, 1979; Bloom, Ghatei, Christofides, Blackburn, Adrian, Lezoche, Basso, Carlei & Speranzo, 1979; Taylor *et al.* 1979; Varner, Modlin & Walsh, 1980; McDonald *et al.* 1981; Ghatei *et al.* 1982) was the fact that there was no detectable rise in plasma gastrin in response to either bombesin or GRP. The finding is the more ironic given that GRP takes its name from this particular activity, which has been well established in other species. In both man and dog gastrin is released in response to bombesin at doses well below that which we have employed in the calf and it therefore seems most likely that the difference is attributable to species variation. CCK proved similarly unresponsive to both bombesin and GRP although it has also been reported to be released in response to bombesin in other species (Fender *et al.* 1976; Miyata, Guzman, Rayford & Thompson, 1978; Ghatei, 1982).

There seem to be genuine species differences in the effects that bombesin-like peptides exert on the pancreatic hormones other than PP. In man, bombesin has been reported to produce a significant rise in plasma glucagon, but not insulin, concentration (Fallucca, Delle Fave, Gambardella, Mirabella, De Magistris & Carratù, 1977; Ghatei, 1982), although the effect is comparatively small and has not been observed in other studies. In the conscious dog I.V. infusions of bombesin have been found to release both glucagon and insulin, but seem to be more potent in effecting the former response (Schusdziarra *et al.* 1980; McDonald *et al.* 1981). In the present study both bombesin and GRP produced an abrupt rise in mean plasma glucagon and insulin concentration and proved to be exceptionally potent in relation to release of the latter. This is attested to by the fact that the peak incremental values 10 min after the infusions had been initiated, 570 ± 120 pmol/l (bombesin) and 556 ± 110 pmol/l (GRP), were roughly three times greater than the rise in mean plasma insulin concentration which occurs over the same period of time in calves of the same age when the plasma glucose concentration is doubled by I.V. infusions of exogenous glucose (Bloom & Edwards, 1981). In spite of the extraordinary insulinotropic potency of these peptides, which are released so readily in response to splanchnic nerve stimulation in this species (Bloom & Edwards, 1982), insulin release is invariably suppressed during splanchnic nerve stimulation in normal conscious calves (Bloom & Edwards, 1980), presumably due to the overriding effect of α-adrenergic inhibition when the whole of the splanchnic sympathetic innervation is stimulated simultaneously.

The fact that bombesin-like immunoreactivity is thought to be restricted to nerve terminals (Dockray, Vaillant & Walsh, 1979) is supported by the observation in the present study that it is present in much higher concentrations in the smooth muscle than it is in the mucosa of the gastrointestinal tract. The finding that infusions of the exogenous peptides, at a dose which reproduces the plasma concentration achieved in response to splanchnic nerve stimulation, themselves produce numerous biological responses strongly suggests that this putative neurotransmitter also acts via the circulation in classical hormonal fashion.

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